PRODUCTION OF POLYSACCHARIDES FROM WHITE MUSHROOMS (*Pleurotus florida*) BY SUBMERGED CULTURE FERMENTATION (SCF)

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ABSTRACT

P.florida is commonly available edible mushroom among different types of oyster mushrooms of the genus *Pleurotus*. The bioactive compound contain in the *P.florida* (polysaccharides and protein) have benefit to health include immunomodulatory, anti-cancer, and hypocholesterolemic effect. The objective of this research is to study the production of polysaccharides and biomass from white mushrooms (*P. florida*) by submerged culture fermentation (SCF) and determination of protein content by using Kjeldahl method. The effect of harvest time on the production of mycelial biomass, exopolysaccharides (EPS) and protein by *P.florida* was investigated in research. The optimal culture condition was 20 g/L glucose, 4 g/L yeast extract, 0.46 g/L KH2PO4, 1 g/L K2HPO4, and 0.5 g/L MgSO4 7 H2O at temperature 28oC and pH 8. The modes of reactor operation used in this cultivation of mushroom are shake flask and air lift bioreactor and Somogyi-Nelson reagent, Phenol-Sulphuric reagent and 4% Phenol reagent were used during the sample analysis. In this present study, the result implies that the highest biomass, polysaccharides and protein yield production change with the time of cultivation. This indicated that the maximum production of production day in producing biomass was at days 15 (2.464 g/L). However, the production polysaccharides and protein indicates the highest productivity on the same day (days 12) with 8.42 g/L and 28.69 % respectively. It was found that productivity in airlift bioreactor much higher than the productivity in the shake flask. Thus it can be concluded that that harvest time selection is very important factor to obtain maximal fungal production. This may due to the gentle agitation and aeration hydraulic in airlift bioreactor leads to the productivity of mycelia biomass, exopolysaccharides and protein more efficient than in shake flask.

ABSTRAK

P.florida adalah antara cendawan yang boleh dimakan diantara pelbagai jenis cendawan tiram daripada genus Pleurotus. Sebatian bioaktif yang terdapat di dalam *P.florida* (polisakarida dan protein) mempunyai manfaat untuk kesihatan termasuk immunomodulatori, anti-kanser, dan kesan hipokolesterolemik. Objektif bagi kajian ini ini ialah untuk mengkaji pengeluaran polisakarida dan biojisim daripada cendawan putih (*P. florida*) dengan melalui kaedah tenggelam penapaian (SCF) dan analisa kandungan protein pula menggunakan kaedah Kjeldahl. Kesan masa menuai kepada pengeluaran biomass mycelial, exopolysaccharides (EPS) dan protein dengan *P.florida* telah dikaji dalam penyelidikan ini. Kultur yang optimum adalah pada 20 g/L glukosa, 4 g/L ekstrak yis, 0.46 g/L KH_2PO_4, 1 g/L K_2HPO_4, dan 0.5g/L $MgSO_4$ 7 H_2O pada suhu 28 ^oC dan pH 8. Mod operasi reaktor yang digunakan dalam pengkulturan cendawan ini adalah kelalang dan bioreaktor angkat udara manakala Somogyi-Nelson reagen, Fenol-Sulphuric reagen dan 4% Fenol reagen telah digunakan semasa analisis sampel. Di dalam kajian ini, hasilnya menunjukkan bahawa biomas tertinggi, polisakarida dan protein berkaitrapat dengan masa penanaman. Ini menunjukkan bahawa pengeluaran maksimum hari pengeluaran dalam menghasilkan biomass adalah pada hari 15 (2,464 g/L). Walau bagaimanapun, polisakarida dan protein pengeluaran menunjukkan produktiviti tertinggi pada hari yang sama (hari 12) dengan 8.42 g/L dan 28.69%. Hasil kajian ini juga mendapati bahawa produktiviti pada mod penyelamatan udara bioreaktor lebih tinggi daripada produktiviti di kelalang. Oleh itu, ia dapat disimpulkan bahawa bahawa pilihan masa menuai adalah faktor penting untuk mendapatkan pengeluaran kulat maksimum dan kerana pergolakan lembut dan pengudaraan hidraulik dalam penyelamatan udara bioreaktor membuat produktiviti biomas mycelia, exopolysaccharides dan protein lebih baik daripada di dalam kelalang.

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LIST OF SYMBOL

°C	Degree Celsius
cm	Centimetre
%	Percentage
g	Gram
mg	Milligram
µg	Microgram
L	Litre
ml	Milliliter
V	volume

LIST OF ABBREVIATIONS

DW	Dry Weight
SSF	Solid State Fermentation
SCF	Submerged Culture Fermentation
EPS	Exopolysaccharides
PPS	Endopolysaccharide

1 INTRODUCTION

1.1 Introduction

Cancer is a leading cause of death worldwide. Based on the report by Ministry of Health Malaysia in 2006, an amount of 21,773 cancer incidence was reported per 1000,000 populations in peninsular Malaysia. It comprises of 9,974 males and 11,799 females. The five most common cancers among population of Peninsular Malaysia in 2006 were breast, cervix colorectal, nasopharynx, and lung. Mushrooms consist of bioactive compound such as polysaccharides and proteins as reported in Huang and Liu (2008). Recently, a number of bioactive molecules, including anti-cancer agents, have been identified from various mushrooms. These bioactive compounds are responsible in anti-cancer effects. Polysaccharides are the best known and most potent mushroom-derived substances with anti-cancer and immunomodulation properties (Daba and Ezeronye., 2003).

Polysaccharides especially β-glucan obtained from mushroom have been used for various purposes. Glucan with different active unit linkage such as (1→3), (1→6)-β-glucan and (1→3)-α-glucans constitute mushroom polysaccharides which perform immunomodultor activity as they are biological response modifiers (BRMs) (Rout *et al*., 2005). Exopolysaccharide are highly molecular weight polymers composed of sugar residue that secreted by microorganism. In both prokaryotic and eukaryotic microbial pathogens, polysaccharides are major cell wall components and act as a source of therapeutic agents which are able to modulate animal and human response and able to inhibit certain tumor growth.

The crude protein content of the common mushroom (*P. florida*) has been reported to be 19-38% on dry weight (DW) basis, but the measurement are complicated by the presence on non-protein nitrogen containing compounds. Protein content of mushroom is influenced by the compost composition, flush number, harvest time and strain (Braaksma *et. al*., 1995).

Submerged culture fermentation (SCF) is an alternative methodology to produce polysaccharides (bioactive compound) from mushrooms at higher yield for nutraceutical, pharmaceutical, and cosmetics industries. Modes of reactor operation that are the most amenable to mycelium production of mushroom is air lift bioreactor due to its gentle agitation and aeration hydraulics.

1.2 Objectives

The current medicine for anti-cancer that available in market pose several side effect and complications in clinical management of various form of cancer which highlights the urgent need for novel effective and less-toxic therapeutic approaches (Patel *et al*., 2012). A few of clinical trial have been conducted in identifying the benefit of using commercial medicine from mushroom extracts in cancer therapy it contains bioactive compounds like protein and polysaccharide. It has a function as anti-cancer agents. In addition, the production of polysaccharides and protein by submerged culture fermentation (SCF) is an alternative way and has advantage to reduce the cost and improve the productivity (Smiderle *et al*., 2011), as compared to the solid state fermentation (SSF), besides more precise control on a range of factors such as pH, moisture, light, and temperature (Lin and Sung, 2006). Hence, this application will be implemented in this present study.

1.3 Research objective

To study the production of polysaccharides and biomass from white mushrooms (*Pleurotus florida*) by submerged culture fermentation (SCF) and determination of protein content by using Kjeldahl method.

1.4 Scope of research

In order to achieve the objectives, the following scopes have been identified:

i. To identify the effect of harvest time on polysaccharides production in shake flask.
ii. To identify the effect of harvest time on mycelium production in shake flask
iii. To identify the effect of harvest time on protein production in shake flask.
iv. To compare the productivity for polysaccharides, mycelium and protein in shake flask and airlift bioreactor at 28^{o}C (lee at al, 2009).

2 LITERATURE REVIEW

2.1 Mushroom cultivation and consumption

2.1.1 Mushroom production

Approximately 14,000 species of the 1.5 million fungi estimated in the world produce fruiting that are large enough to be considered as mushrooms. There are three categories of mushrooms available in industries which are edible mushrooms, medicinal mushrooms products, and wild mushrooms (Chang, 2006). In Far East, over 200 species have been collected from the wild and used for various traditional medical purposes. About 20 species have been cultivated on industrial scale and 35 species have been cultivated commercially. The most cultivated mushroom worldwide is *Agaricus bisporus*, followed by *Lentinula edodes*, *Pleurotus* spp. and *Flammulina velutipes* (Aida *et al*., 2009) & (Chang and Miles, 2004). These species require shorter growth time when compared to other edible mushrooms, they demand few environmental controls, and they can be cultivated in a simple and cheap way (Bonatti *et al*., 2004). Table 2.1 shows summarization world mushroom production taken from Food and Agriculture Organization of United Nations (2009). China was found be the biggest production of mushrooms as they produced more than 1.5 million tonnes in 2007. This was followed by United Stated of America as the second highest world production mushrooms. However, Indonesia and India producing least mushroom this maybe due to less demand of mushrooms in the country.

Table 2.1 Summarize World mushroom Production, 1961-2007; United Nations (Food and Agriculture Organization of United Nations, 2009).

Countries	Production (tonnes) 2007
China	15,68,523
United States of America	3,59,630
Netherlands	2,40,000
Indonesia	48,247
India	48,000
Other countries	59,297

2.1.2 *P. florida*

Pleurotus species are commonly called Oyster mushrooms. There are about 40 species of this mushroom. Oyster mushrooms now rank second among the important cultivated mushrooms in the world. *P.florida*, the oyster mushroom, is a common edible mushroom. It was first cultivated in Germany as a subsistence measure during World War I and is now *P.florida* is widely cultivated and consumed as food in Malaysia. The scientific classification of *P.florida* is stated as in Table 2.2.

Table 2.2 Classification of *P.florida* (http://en.wikipedia.org/wiki/Pleurotus)

Kingdom	Fungi
Phylum	*Basidiomycota*
Class	*Agaricomycetes*
Order	*Agaricales*
Family	*Pleurotaceae*
Genus	*Pleurotus*
Species	*P. florida*
Binomial name	*Pleurotus florida*

Figure 2.1: Fruit body of *P. florida.*

2.2 Medicinal and pharmaceutical properties of mushroom

2.2.1 Anti-cancer properties of mushrooms

Cancer is a leading cause of death worldwide. The current medicine for anti-cancer that available in market pose several side effect and complications in clinical management of various form of cancer which highlights the urgent need for novel effective and less-toxic therapeutic approaches (Patel *et. al*., 2012). A few of clinical trial have been conducted in identifying the benefit of using commercial medicine from mushroom extracts in cancer therapy. Mushrooms consist of bioactive compound such as polysaccharides, proteins, fats, ash, glycosides, alkaloids, volatile oils, tocopherols, phenolics, flavonoids, carotenoids, folates, ascorbic acid enzymes, and organic acids. These bioactive compunds, are responsible in anti-cancer effects. Recently, a number of bioactive molecules, including anti-cancer agents, have been identified from various mushrooms. Polysaccharides are the best known and most potent mushroom-derived substances with anti-cancer and immunomodulation properties. Figure 2.2 shows structure of anti-cancer compounds isolated from mushrooms.

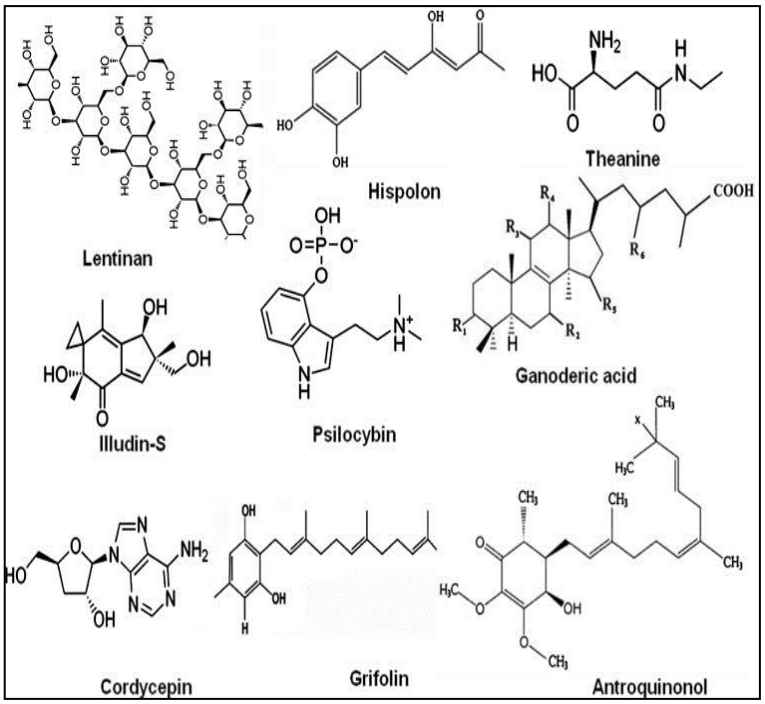

Figure 2.2 Structure of anti-cancer compounds for mushrooms (Patel *et. al*.,2012)

2.2.2 Structure and anti-cancer activities of polysaccharides

Polysaccharides belong to a structurally diverse class of macromolecules. Unlike proteins and nucleic acids, they contain repetitive structural features which are polymers of monosaccharide residues joined together by glycosidic linkages with a general chemical formula of $C_x(H2O)_y$. Research done by Maity, (2013) found that mushroom (*P. florida)* polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as water-insoluble (1→3)(1→6)-β-D-glucans, water-soluble (1→6)-α-D-glucan, NaCl soluble (1→3)(1→6)-α, β-D-glucan a water-soluble heteroglycan are widely used as anti-cancer and immunostimulating agent. Table 2.3 shows the polysaccharides isolated from the parent strains. Polysaccharides play important roles in biological mechanisms such as infection, adhesion, immune response and signal transduction. In addition, mushroom polysaccharides exert their anti-cancer action through activation of immune response (Wasser and Weis, 1999) and these substances are regarded as biological response modifiers. Figure 2.3 shows the structure of repeating unit present in polysaccharides.

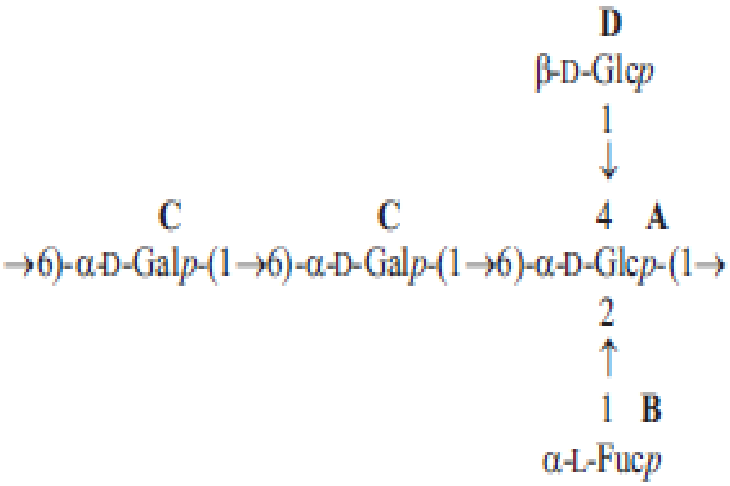

Figure 2.3 The structure of the repeating unit present in the polysaccharides (Maity et al., 2011).

Table 2.3 The polysaccharides isolated from the parent strains (Wasser., 2002).

Parent strain	**Polysaccharide**
P.florida	(1-6)-a-D-Glucan (from hot aqueous extract),
	(1-3), (1-6)-a-, b-D-Glucan (from NaCl soluble Fr. I)
	(1-3), (1-6)-b-D-Glucan (water insoluble)
	(1-3), (1-6)-b-D-Glucan (from cold alkaline extract)

2.2.3 Determination protein in mushrooms

Mushrooms have been considered as a source human healthy food for a long time ago (Chang and Miles., 1992). Mushrooms are rich in carbohydrate and protein and the protein elaborated by these fungi have shown several biological activities like immunomodulatory, anti viral, antiproliferative, antifungal and anti bacterial effects (Jonathan and Fasidi., 2003).

Protein content in mushrooms is influenced by the flush number, harvest time (Crissan and Sands., 1978), and compost composition (Bakowski *et al.,* 1986). Protein content is often expressed on dry weight (DW) basis, but DW is not constant and is reported to be variable between 7.4 to 13.7% (Weaver et al., 1977). There is a few of research report that the total protein content of harvested mushrooms in range between 19 to 39 % on

DW basis (Weaver et al., 1977). It also suggested that the crude protein content in mushrooms arises by the presence of nitrogen determination (Kjeldahl) and usually attributed to protein. The protein is then determined on the basis of total nitrogen content, a Kjeldahl factor of N x 4.38 is commonly used (Levai, 1989).

The Kjeldahl method process include the digestion of the sample by place the digestion tablets into the Kjeldahl tube. Next process involve was the distillation of the sample followed with the titration and the calculation of the crude protein with Kjeldahl factor of N x 4.38. (Riddellová, undated). Figure 2.4 shows the digestion method involves in the determination of protein content in *P.Florida.*

Figure 2.4: Digestion process in Kjeldahl method

2.3 Technique in mushroom cultivation

2.3.1 Solid State Fermentation (SSF)

Many different techniques and substrates have been successfully utilized for mushroom cultivation. For production of mushroom fruiting bodies, various forms of SSF are employed, whereas for mycelial biomass and polysaccharides production, submerged fermentation is preferable to produce a more uniform biomass and pharmaceutical products.

Solid-state fermentation (SSF) is defined as any fermentation process occurring in the absence or near absence of free liquid, employing an inert substrate (synthetic materials) or a natural substrate (organic materials) as a solid support (Pandey et. al., 2000 and Couto et. al., 2007). Figure 2.5 shows the solid state fermentation in *P.Florida* production. SSF is most appropriate for bioconversion of plant raw materials into value-added products, such as mushroom fruiting bodies, fodder, secondary metabolites, and enzymes. SSF has several advantages as compared with submerged cultivation; in particular, with small energy consumption, the nutrient medium is concentrated, and high volumetric productivity can be achieved in a smaller bioreactor. In addition, till now, the major obstacles for the commercial applications of SSF techniques have not been completely overcome. They are related to the design and operation of large-scale bioreactors due to problems concerned with the control of parameters such as pH, temperature, aeration and oxygen transfer, moisture, and agitation.

Figure 2.5: Solid State Fermentation (SSF) in mushroom cultivation.

2.3.2 Submerged culture fermentation (SCF)

In submerged culture fermentation (SCF) is different with SSF where it requires large energy expenditures to agitate nutrient medium and to supply oxygen. However, the submerged culture works as a homogeneous system, and the cultivation process control is easy using many on line sensors. In this case, a very wide range of products can be produced from a wide range of microorganisms with the best productivity, due to medium mixing and unlimited diffusion of nutrients. Moreover, although the downstream processing after submerged fermentation requires removal of large volumes of water and is more expensive, the product purification may be easier as compared to SSF. Hence, mushroom submerged cultivation has significant industrial potential, but its success on a commercial scale depends on cost compared with existing technology (Pandey. et. al., 2000). Figure 2.6 shows the submerged culture fermentation (SCF) in mushroom cultivation in the shake flask.

Figure 2.6: Submerged Culture Fermentation (SCF) in mushroom cultivation.

2.3.3 Submerged culture in airlift bioreactor

In mushrooms cultivation, the productivity in an airlift reactor was higher than that in the stirred-tank reactor and shake flask (Cho et al., 2006). In airlift fermenter, mixing is accomplished without any mechanical agitation. Airlift bioreactor is suitable to be used for tissue culture because tissue is shear sensitive and normal mixing is not possible. Since there is no agitation, sterility can easily be maintained (Najafpour, Undated). Research done by Kim et al., (2002) has mentioned that the mycelial biomass productions were 3 times and 2 times higher than those in shake cultures, respectively. Lee et al., (1998) have reported that maximum polysaccharide production and mycelial biomass of the same species of mushroom. This proved the effectiveness of mushroom cultivation in airlift bioreactor. Figure 2.7 shows the cultivation of mushrooms in airlift bioreactor.

Figure 2.7: Mushrooms cultivation in airlift bioreactor.

3 MATERIALS AND METHODS

3.1 Material used

3.1.1 Fungi (Mushrooms)

The raw material used in this study was *P. florida* (white mushroom). It was purchased from the local hypermarket. The mushrooms were transported to the laboratory and placed in the freezer with temperature 20ºC to reduce any protease activity before handling the experiment.

3.1.1 Chemicals

For the analysis of polysaccharides, Somogyi Nelson reagent, Arsenomolybdate reagent and 4% Phenol reagent was used. For the Somogyi-Nelson reagent, the copper sulphate pentahydrate, anhydrous sodium carbonate, sodium hydrogen carbonate and sodium tartarate tetahydrate were purchased from Sigma Aldrich (Malaysia). While for Arsenomolybdate reagent, ammonium molybdate, concentrated sulphuric acid and disodium hydrogen arsenate heptahydrate were also purchased from Sigma Aldrich (Malaysia). Besides, 4% Phenol reagent also purchased from Sigma Aldrich (Malaysia).

3.2 Experimental procedure

For the analysis of polysaccharides, Somogyi Nelson reagent, Arsenomolybdate reagent and 4% Phenol reagent was used. For the Somogyi-Nelson reagent, the copper sulphate pentahydrate, anhydrous sodium carbonate, sodium hydrogen carbonate and sodium tartarate tetahydrate were purchased from Sigma Aldrich (Malaysia). While for Arsenomolybdate reagent, ammonium molybdate, concentrated sulphuric acid and disodium hydrogen arsenate heptahydrate were also purchased from Sigma Aldrich (Malaysia). Besides, 4% Phenol reagent also purchased from Sigma Aldrich (Malaysia).

3.2.1 Somogyi-Nelson Reagent preparation

The first solution was prepared by mixed 4 g of copper sulphate pentahydrate and 16 g sodium hydrogen carbonate into 200 ml distilled water. Second solution prepared with 24 g anhydrous sodium carbonate and 12 g sodium tartarate tetahydrate diluted in 250 ml distilled water. Separately, prepare a solution of 180 g anhydrous sodium sulfate in 500 ml of boiling distilled water. The two solutions were combined in a volumetric flask and diluted the final solution to 1 liter (Winstad, 2001).

3.2.2 Arsenomolybdate Reagent preparation

A solution of 25 g ammonium molybdate in 450 ml distilled water was prepared. The solution was added with stirring 21 ml concentrated sulphuric acid and 25 ml of distilled water containing 3 g disodium hydrogen arsenate heptahydrate. The solution was stirred continuously for 24 hours at 37ºC. The solution was stored in a 1 liter glass stoppered brown bottle and covered with aluminum foil to protect the solution from light (Winstad, 2001).

3.2.3 4% Phenol Reagent

40 g phenol was added in 1 litre distilled water (Winstad, 2001).

3.2.4 Tissue culture preparation

A piece of stem from fruit body *P.florida* was sterilely removed and placed in potato dextrose agar (PDA) plate. The sample was inoculated in the PDA agar for a few days in room temperature. After 10 days, mycelium grows from the tissue and colonized the agar. The tissue was then gently cut into small pieces (1 cm x 1 cm) before inoculated into the 500 ml shake flasks that contain mushroom complete medium (MCM).

3.2.5 Preparation of Mushroom Complete Media (MCM)

Add 20 g of glucose, 2 g yeast extract, 2 g meat peptone, 1 g K_2HPO_4, 0.46 g HK_2PO_4 and 0.5 g $MgSO_4.7H_2O$. After that, mix the chemicals with 1 liter distilled water. Then, divide the volume to 250 ml each and transfer to shake flask. Lastly, the media was autoclaved in 121ºC in 15 minutes (Winstad, 2001).

3.2.6 Submerged Culture Fermentation (SCF)

The samples were then left in 1 day in room temperature to grow before introduced into the shaker. The incubation was conducted on a rotary shaker at 25℃, 500 rpm for 6 days, 9 days, 12 days, 15 days and 18 days. For production in airlift bioreactor, the sample was fermented in shake flask first before being transferred in the reactor and was left in 7 days. The determinations of endopolysaccharides are by freeze dry of the biomass before weight the sample while the determination of exopolysaccharides has been done as below (Winstad, 2001).

3.3 Analysis

3.3.1 Determination of Reducing Sugar Using the Somogyi -Nelson Method

The samples were diluted first with the dilution factor 10 x. A portion of sample (1 ml) then transferred into 10 ml test tube. 1 ml of low-alkalinity copper reagent added and the tubes was heated in boiling water for 10 min. 1 ml of arsenomolybdate reagent was then added to the tubes and filled with the distilled water up to 5 ml. The sample was left in 15 minutes at room temperature. The solution transferred from the test tubes to cuvettes and the sugar measured at the Absorbance 500 by using spectrophotometer (Winstad, 2001).

3.3.2 Determination of reducing and non-reducing (total) sugar using the Phenol-Sulfuric Assay method

The samples were diluted first with dilution factor 10 x. Then, 1 ml of sample then transferred to 10 ml tube before 500 µl of 4% phenol was added. 25 ml of 90% sulphuric acid was then added into the sample and mixed well the sample with vortex. Then the solution transferred from the test tubes to cuvettes and the sugar measured at the Absorbance 490 by using spectrophotometer (Winstad, 2001).

3.3.3 Determination of Protein Using Kjeldahl Method

The protein content in the *P.florida* was analyzed in term of total nitrogen which is Kjeldahl method. The biomasses of all samples were dried first using freeze dryer. 0.2 mg of biomass were insert into the Kjeldahl tube and the Kjeldahl tablet were added. 10